Metal accumulation and its effect on leaf herbivory in an allopolyploid species Arabidopsis kamchatica inherited from a diploid hyperaccumulator A. halleri

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DOI: https://doi.org/10.1111/1442-1984.12304

Received: 21 July 2020 | Revised: 25 September 2020 | Accepted: 5 October 2020

DOI: 10.1111/1442-1984.12304

ORIGINAL ARTICLE

PLANT SPECIES BIOLOGY WILEY

Metal accumulation and its effect on leaf herbivory in an allopolyploid species *Arabidopsis kamchatica* inherited from a diploid hyperaccumulator *A. halleri*

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Funding information
Japan Society for the Promotion of Science, Grant/Award Numbers: 16H06469, 18H04785; Japan Science and Technology Agency: JPMJCR16O3: JPMJPR15Q7: JPMJPR17Q4; Swiss National Science Foundation: 31003A_182318; University of Zurich, Grant/Award Number: University Research Priority Programs (URPP)

Abstract

Excessive amounts of metal ions in soil are toxic for most plant species, yet metal can also facilitate plant survival by elemental defense against herbivores and pathogens. Zinc and cadmium hyperaccumulation in *Arabidopsis halleri* is known to be effective for the defense against natural enemies. The allotetraploid species *A. kamchatica*, derived from *A. halleri* and a non-hyperaccumulator *A. lyrata*, has a lower hyperaccumulation level of zinc than *A. halleri*, but its significance for elemental defense remains unknown. In this study, we evaluated the accumulation levels of zinc and cadmium in the allotetraploid compared with its diploid progenitors, and evaluated the contribution of metal treatments to anti-herbivore resistance under field conditions. The accumulation level of zinc in *A. kamchatica* was intermediate between the progenitors, but that of cadmium was lower than in both diploid progenitors. The elemental defense of *A. kamchatica* and *A. halleri* was supported by a field experiment comparing the herbivory level between a control group and metal-supplemented plants. Moreover, the effect of elemental defense was lower in *A. kamchatica* than in the hyperaccumulator progenitor *A. halleri*, which is consistent with the metal accumulation level. This result reveals that the allotetraploid plant inherited its hyperaccumulating ability from one progenitor as an advantageous trait but at an intermediate level.

KEYWORDS

allotetraploid, *Arabidopsis kamchatica*, elemental defense, herbivory, metal hyperaccumulation

1 | INTRODUCTION

Metal hyperaccumulation is a trait that has evolved recurrently in distantly related plant species (Krämer, 2010). Even though the origin of this trait is still under much debate, it is adaptive to metalliferous soil, which comprises multiple steps: uptake of heavy metals in soil via roots, transporting them to shoots, and detoxifying by sequestering them in vacuoles. Approximately 500 species are hyperaccumulators of one or more metal elements of Zn, Cd, Ni, Se, As, and others, at levels that are toxic for common plant species (Krämer, 2010).

Besides their detoxifying mechanism underpinning their tolerance to metalliferous soil conditions (Krämer, 2010; Rascio & Navari-Izzo, 2011), hyperaccumulators are known to also utilize the toxicity of metals for their own benefit. According to this hypothesis, known as "elemental defense", a hyperaccumulator uses metals obtained from the soil as a defense against its natural enemies, such as herbivores and pathogens (Boyd, 2007 and the references included therein; Hörger, Fones, & Preston, 2013; Kazemi-Dinan, Thomaschky, Stein, Krämer, & Müller, 2014; Ribeiro et al., 2017). This defensive effect could even be strengthened by the combination of multiple metal elements (Boyd, 2012; Kazemi-Dinan et al., 2014).

Among many distant taxa containing hyperaccumulator members as a result of parallel evolution, the family Brassicaceae is recognized for containing the highest number of hyperaccumulator species (Krämer, 2010). Among them, *Arabidopsis halleri* has served as a model plant species for studying the genetic mechanisms of hyperaccumulation. Many genes and chromosomal loci contributing to zinc and cadmium tolerance have been identified by quantitative trait loci mapping (Courbot et al., 2007; Dräger et al., 2004; Willems et al., 2007) and comparative genomics (Becher, Talke, Krall, & Krämer, 2004; Talke, Hanikenne, & Krämer, 2006; Weber, Chételat, Reymond, & Farmer, 2004) between *A. halleri* and its non-hyperaccumulator sibling species, *A. lyrata*, revealing a key role of metal transportation in hyperaccumulators. In addition to these genetic studies, *A. halleri* has been also used to study the ecological consequences of hyperaccumulation. Indeed, a study using Zn- and Cd-supplemented *A. halleri* plants demonstrated the effect of elemental defense against insect herbivores (Kazemi-Dinan et al., 2014). These lines of molecular and ecological evidence suggest that elemental defense is an adaptive trait in *A. halleri*.

The hyperaccumulator *A. halleri* is also known as a progenitor of the allotetraploid species, *A. kamchatica*, with another diploid progenitor, *A. lyrata*. The contrasting metal accumulation abilities of these progenitors were shown to produce an intermediate level of zinc accumulation in the allotetraploid (Paape et al., 2016, 2020), but how the polyploidization affects its elemental defense has not been evaluated yet. A recent population genetic analysis of the tetraploid *A. kamchatica* using the genome-wide SNP data from 25 populations covering its distribution range detected a lower nucleotide diversity at one of the key genes for hyperaccumulation, suggesting a signature of selective sweep on *A. halleri* homeologs but not on *A. lyrata* homeologs (Paape et al., 2018). These genetic findings thus make *A. kamchatica* a unique example for studying how a polyploid species inherits an ecologically beneficial trait from its progenitors. Moreover, the geographically wider distribution of *A. kamchatica* compared with its progenitors (Shimizu-Inatsugi et al., 2009) and its high intraspecific genetic variation (Paape et al., 2018) offer an excellent opportunity to study how an ecologically beneficial trait in a polyploid species undergoes differing selective pressure among variable environments.

On the other hand, herbivory stress on *Arabidopsis* species has been intensively studied, in both ecological and molecular contexts (reviewed by Poecke, 2007). Field studies with *A. thaliana* have revealed a wide array of herbivore species feeding on it, highlighting the importance of combining field (or *in natura*) and laboratory studies to better understand plant–insect interactions (Kudoh, 2016; Sato et al., 2019; Sato, Shimizu-Inatsugi, Yamazaki, Shimizu, & Nagano, 2019; Shimizu, Kudoh, & Kobayashi, 2011). Both progenitors of *A. kamchatica* are perennial species which are attacked by a wide taxa of insect herbivores, including coleopterans (Kawagoe, Shimizu, Kakutani, & Kudoh, 2011; Sato, 2018; Sato & Kudoh, 2017), lepidopterans (Løe, Toräng, Gaudeul, & Ågren, 2007; Sato & Kudoh, 2017; Sletvold, Huttunen, Handley, Kärkkäinen, & Ågren, 2010) and hemipterans (Sato & Kudoh, 2017; Yano, 1994) in wild populations across the year. In particular, the hyperaccumulator *A. halleri* is attacked by flea beetles (*Phyllotreta* spp.) in late spring and early autumn (Sato & Kudoh, 2015). Previous studies have shown that accumulation of Zn and Cd in *A. halleri* can deter leaf-chewing and sap-sucking herbivores (Kazemi-Dinan et al., 2014; Stolpe, Krämer, & Müller, 2017). However, it remains unknown whether the allotetraploid species *A. kamchatica* inherited this ability against insect herbivores.

The purpose of this study was to evaluate the effect of the elemental defense hypothesis in an allotetraploid of a hyperaccumulator plant species using a combined zinc and cadmium treatment. Specifically, we addressed two questions. (i) Has the allotetraploid *A. kamchatica* inherited the elemental defense trait from *A. halleri*? (ii) Is the level of elemental defense in *A. kamchatica* intermediate between the two progenitors? We conducted a field experiment comparing the herbivory level between *A. kamchatica* and its diploid progenitors with metal-amended soil, and discussed how their metal accumulation level and the defense effect are related.

TABLE 1 Detailed description of plant origin and ploidy

Genotype ID	Species	Ploidy	Trichome	Original locality	Locality description
hal1	*Arabidopsis halleri*	2*x*	No	Sieber, Herzberg am Harz, Niedersachsen, Germany	Mining site
hal2	*Arabidopsis halleri*	2*x*	No	Bode, Lenk in Simmental, Bern, Switzerland	Non-contaminated site
lyr1	*Arabidopsis. lyrata*	2*x*	No	Siberia, Russia	
lyr2	*Arabidopsis lyrata*	2*x*	No	Oyotung, Indigirka River, Russia	
kam1	*Arabidopsis kamchatica*	4*x*	Hairy	Murodo, Toyama, Japan	Mountainous region
kam2	*Arabidopsis kamchatica*	4*x*	Hairy	Takashima, Lake Biwa, Shiga, Japan	Sandy lakeshore

2 | MATERIAL AND METHODS

2.1 | Plant genotypes and preparation

We used two genotypes of each diploid species: *Arabidopsis halleri* subsp. *halleri* and *Arabidopsis lyrata subsp. petraea*, and the allotetraploid *Arabidopsis kamchatica* (one of subsp. *kamchatica* and one of subsp. *kawasakiana*). The origin, genotype ID name and the trichome phenotype of the six genotypes are summarized in Table 1. The two genotypes of *A. halleri* are from contrasting habitats of a mining site (hal1) and non-mining site (hal2), respectively. The two *A. lyrata* genotypes are from distinct populations in far east Russia (lyr1 and ly2). The two genotypes of *A. kamchatica* have different origins of polyploidization, being derived from a mountainous region (kam1) and sandy lakeshore (kam2), respectively.

For *A. lyrata* and *A. kamchatica* genotypes, seeds were collected from selfed mother plants in the laboratory and were left to germinate on hydrated sand in a plastic dish. The small seedlings were transferred to single pots with the soil as described below. For *A. halleri* genotypes, an outcrossing species, we took small clonal plantlets emerging on the flower stem of the mother individual to transfer them directly onto the soil in single pots.

We incubated 240 plants individually in a 7 × 7 × 6 (length × width × height, cm) pot containing 170 g of commercial soil ("Profi Substrat Typ ED73", Einheitserde, Sinntal-Altengronau, Germany) in a climate chamber, with a 16-h light/8-h dark cycle at 22°C/20°C and 60% RH (relative humidity). The seedlings were incubated for 6 weeks without supplemental metals. We then started the metal treatment by adding the stock solution containing zinc ($ZnCl_2$) and cadmium ($CdCl_2$) to the soil, so that the final concentrations of Zn and Cd in the soil became 300 and 5 μg g^{-1} of hydrated soil, respectively, according to a previous experiment with *A. halleri* (Kazemi-Dinan et al., 2014). At the timing of leaf sampling and the start of the herbivory experiment below, the 240 plants were assigned to 20 randomized blocks, where each block contained the six genotypes with control and metal supplemented conditions.

2.2 | Metal concentration measurement

After 2 weeks of the metal treatment, we collected one mature leaf from 10 replicates of the six genotypes with the two conditions (=120 plants in total) to measure the metal concentration. After digesting the air-dried leaves with 13 N nitric acid, the respective Zn and Cd concentrations in the sample solution were measured by an inductively coupled plasma mass spectrometer (ICP-MS) (NexION 350S, PerkinElmer, Waltham, Massachusetts, USA). The metal amount was normalized by leaf dry weight. The original measurement data are attached as supplementary information (Dataset S1).

2.3 | Herbivory experiment in the field

After 8 weeks of incubation (6 weeks without metal and 2 weeks with metal), 20 replicates (plants) of the six genotypes and two conditions (control vs. treatment with Zn and Cd admixture) were transferred into the common garden at the Irchel Campus of the University of Zurich, Zurich (47°23′N, 8° 33′E) on July 11, 2018. The garden floor was covered with concrete tiles to prevent weeds, except for the experimental plots filled up with soil and covered by plastic woven sheets. At the garden site, flea beetles, aphids, thrips and other insect herbivores potentially occur on *Arabidopsis* plants (Sato, Shimizu-Inatsugi, Yamazaki, Shimizu, &

Nagano, 2019). In the vicinity of these plants, approximately 1,000 *A. thaliana* individuals were incubated for another experiment (ca. 5 m away). No other *Brassica* species were cultivated in the neighborhood during the experimental period.

On the day before moving them to the field, we measured the rosette diameter of all individuals. In the field, 20 blocks were set up; each consisted of 12 individuals (= 6 genotypes × 2 conditions), whose position was randomized in a 3 × 4 distribution (Figure S1). In total, 240 plants were placed in a rectangular shape in a 2 × 10 block layout. The plants were covered with shading cloth (5% transparency) for 4 days, from 13:00 to 17:00, to prevent any damage from direct sunlight before their acclimation (Figure S1). In the field, the number of holes and the number and type of herbivorous insects found on each plant were recorded in the first, second and third weeks. In the fourth week, we evaluated the viability of plants using four categories: dead = 0, irreversibly sick (dry or yellow) = 1, less healthy (yellowish) = 2 and healthy = 3. All data obtained from the census are in the supplementary information (Dataset S1).

2.4 | Statistical analyses

Leaf herbivory, metal accumulation and survival rate were analyzed using generalized linear models (GLMs) or generalized linear mixed models (GLMMs) (Bolker et al., 2009). The response variables were the number of leaf holes, metal concentration (continuous variable) and survival rate (rank variable). We used the Poisson family with a log link function for the count variable (i.e., the number of holes), an ordered multinomial with a logit link function for the rank variable (score of survival rate), and the Gaussian family with an identity link function for the continuous variables (metal concentrations). We conducted species-level and genotype-level testing with the metal treatment incorporated as another explanatory variable. For the species-level testing, plant species and genotype ID were included as fixed and random effects, respectively. For the genotype-level testing, the genotype ID was considered an explanatory variable; for field experimental data, we considered the experimental block ID as a random effect. The initial diameter of each plant individual was included as an offset term in the Poisson GLMMs, to adjust for the effect of plant size on total number of leaf holes. The leaf-level metal quantification prior to transplantation did not significantly affect the number of leaf holes recorded (GLMM, Wald test, $p = .11$); hence, this factor was omitted as an explanatory variable. Statistical differences between the metal treatment and control groupd within each genotype or species were tested using the Tukey's multiple comparisons of means. When a significant difference was detected between two groups' means, the effect size was evaluated by its *Z*-value (= mean difference divided by standard error). All statistical analyses were performed using R v3.5.1 (R Core Team 2018) with the *lme4* (Bates et al. 2015), *ordinal* (Christensen 2019) and *lsmeans* (Lenth 2016) packages. Complete multiple comparisons are available as supplementary tables (Table S1 to S4).

3 | RESULTS

3.1 | Plant growth and metal accumulation in leaf tissue

The size of individuals was evaluated prior to the start of the herbivory experiments by measuring rosette diameter, in order to see the effect of 2 weeks of metal treatment on growth. We did not find any significant difference in size at species or genotype levels (Figure S2), suggesting that the metal treatment did not affect the growth in this period in the climate chamber.

At the same time, we examined the metal accumulation level in the leaf tissue (Figure 1). The two *A. halleri* genotypes showed the highest accumulation of Zn when treated, with levels at a similar range to those reported in previous experiments (Kazemi-Dinan et al., 2014; Paape et al., 2016, 2020). Zn accumulation was detected even in control plants, most probably because the low concentration of zinc in the original soil was actively taken up and concentrated in the leaf tissue. The Zn accumulation of treated *A. lyrata* was ca. 5% that of *A. halleri*. The accumulation in *A. lyrata* without treatment was much lower than that in *A. halleri*. Statistical analysis among the species showed that the zinc level was increased in both *A. halleri* and *A. kamchatica* by the treatment, but that of *A. lyrata* did not show any significant change (Table 2). An intermediate level of Zn accumulation in the tetraploid compared with the diploids was revealed by the magnitude of effect sizes among the three species with or without the metal treatment (Table 2). The two *A. halleri* genotypes showed statistically different Zn accumulation levels, being the highest nonetheless, whereas the difference between the two tetraploid genotypes was smaller (Figure S3a and Table S1). In contrast to Zn accumulation, the Cd accumulation level in *A. kamchatica* was not intermediate between its two progenitors: accumulation in *A. lyrata* was ca. 30% compared with *A. halleri*, but lowest in *A. kamchatica*. Accumulation levels of Cd were variable within species (i.e., between two genotypes of *A. lyrata*

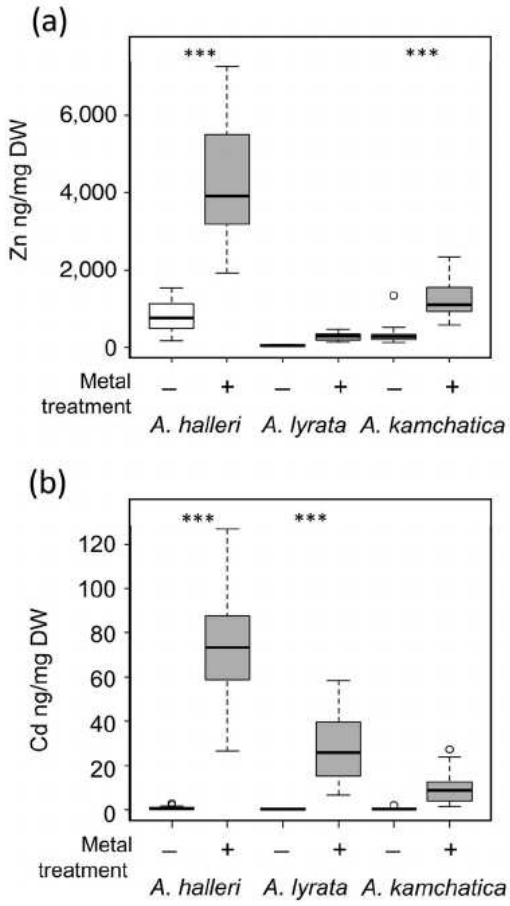

FIGURE 1 Zinc (a) and cadmium (b) accumulation levels in mature rosette leaves. Boxplots for the level of control (−) and metal-supplemented (+) *Arabidopsis* individuals in a mature leaf normalized by its dry weight (DW). The boxplots show: center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; points, outliers. Combinations with a *p*-value <.0001 are marked by ***. ($N = 20$ plants per species × treatment combination)

and *A. halleri*) (Figure S3b and Table S1). Other metals, such as Fe and Mn, were also detected but their accumulation was much lower than that of Zn and Cd (Dataset S1).

Plant viability was recorded in the fourth week in the field (Figure S4; Table S2, S3). Under the metal treatment, *A. lyrata* showed a high mortality rate, consistent with its susceptibility to metal as a non-hyperaccumulator, whereas the survival rate of *A. halleri* was not affected. Yet, the proportion of survived individuals was slightly but significantly higher in the metal-supplemented *A. kamchatica* than in control individuals (Figure S4a; Table S2).

3.2 | Herbivory level in the field experiment

On each census, we found yellow-striped flea beetles (*Phyllotreta striolata*) and black turnip flea beetles (*P. atra*) at low frequency without any other leaf-chewing insects. These flea beetles are oligophagous on Brassicaceae plants and distributed across the world as agricultural pests (Ahuja, Rohloff, & Bones, 2010), and observed in the common garden every year during the growing season. We observed 21 adult beetles in total during the first and second weeks, and six beetles in the third week (Dataset S1). Because the beetles jumped within and among individual plants, making small holes in the leaves, we counted these holes as a proxy for susceptibility to herbivory of each plant. After the second week, we could detect larger areas of feeding damage most probably by other insects, but on only 33 individuals among 240 in the third week, which is not an effective number for statistical analysis.

TABLE 2 Species-level comparisons of Zn and Cd accumulation of three *Arabidopsis* species

Pair	Estimated difference	*SE*	*Z*-value	Adjusted *p*-value
Zn				
A. halleri− − *A. halleri*+	−3,308	183	−18.10	<.0001
A. lyrata− − *A. lyrata*+	−239	185	−1.29	.20
A. kamchatica− − *A. kamchatica*+	−920	183	−5.03	<.0001
Cd				
A. halleri− − *A. halleri*+	−72.68	3.36	−21.65	<.0001
A. lyrata− − *A. lyrata*+	−27.80	3.40	−8.17	<.0001
A. kamchatica− − *A. kamchatica*+	−9.10	3.36	−2.71	.0067

Notes: Least-square means of linear mixed models are given on the scale of raw data, where their estimated differences, standard error (*SE*), Z-value and adjusted *p*-value are shown in the table.
The sign after the sample ID "+" or "−" indicates the metal treatment or control, respectively. The genotype ID was considered a random effect in the mixed models.
p-values were adjusted by Tukey's honest significance difference.

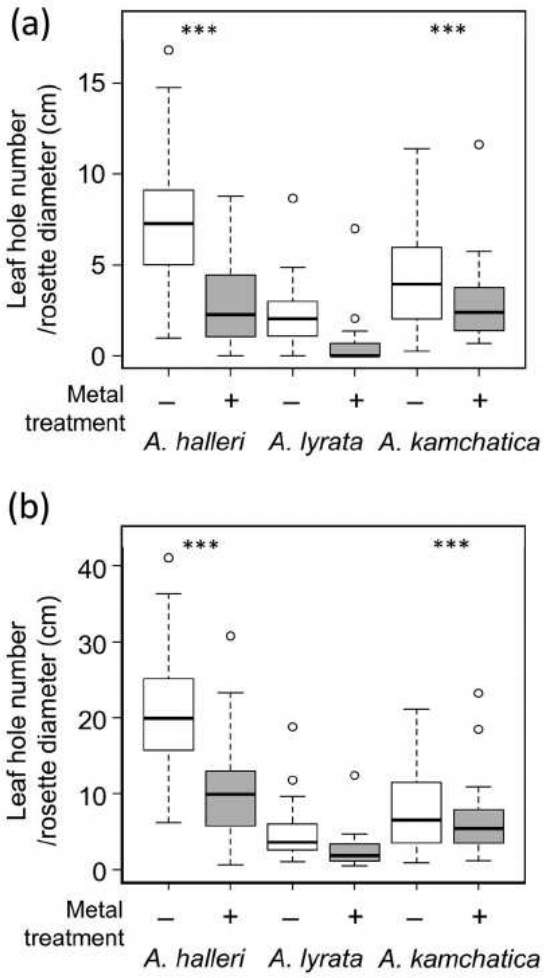

FIGURE 2 Herbivory levels in the field experiment. Boxplots for the number of holes on whole rosette leaves, counted at the second (a) and third (b) weeks of the field experiment, and normalized by the initial rosette diameter. Combinations with a *p*-value <.0001 are marked by ***. The *Arabidopsis lyrata* samples were excluded from the statistical analysis due to their poor viability after the metal treatment and too few holes. ($N = 40$ plants per species × treatment combination)

Because of this situation, we finally statistically analyzed hole numbers in the second and third weeks as an indicator of the intensity of herbivory from flea beetles to evaluate the effect of the metal treatment for each species. The detailed census results are given in Dataset S1.

The defensive effect of metal was evident in the allopolyploid *A. kamchatica*, as well as in the diploid *A. halleri*, according to the GLM results (Figure 2 and Table S3). The effect of metal treatment in *A. lyrata* could not be statistically tested because of its lower survival rate in the metal-supplemented group. The effect size of the metal treatment was smaller in *A. kamchatica* than *A. halleri*, both in the second and third weeks (Table 3), indicating a reduced effect of metal accumulation on herbivory defense in the allopolyploid. When the genotypes were analyzed separately, the number of holes was significantly lower in metal-supplemented groups of all four genotypes in the second week. In the third week, the largest metal treatment effect occurred in the hal2 plants, with those of hal1 and kam1 having lower effect sizes, whereas no metal treatment effect was found for kam2 (Figure S5 and Table S4).

4 | DISCUSSION

4.1 | Intermediate metal accumulation level of Zn, but not Cd, in the allotetraploid

The allotetraploid *A. kamchatica* accumulated approximately half the amount of Zn found in the hyperaccumulator diploid *A. halleri*. However, the accumulation of Cd in *A. kamchatica* was not intermediate, but actually the lowest, among the three congeneric species. According to the quantitative loci analysis of metal accumulation using the F2 progeny of *A. halleri* and *A. lyrata* (Willems et al., 2010), the

TABLE 3 Species-level comparisons of the number of leaf holes in the second and third weeks in the field of three *Arabidopsis*

Pair	Estimated difference	*SE*	*Z*-value	Adjusted *p*-value
Second week				
A. halleri− − *A. halleri*+	0.91	0.032	28.36	<.0001
A. kamchatica− − *A. kamchatica*+	0.47	0.034	13.60	<.0001
Third week				
A. halleri− − *A. halleri*+	0.70	0.019	37.44	<.0001
A. kamchatica− − *A. kamchatica*+	0.23	0.024	9.73	<.0001

Notes: Least-square means of a generalized linear mixed model (GLMM) with a Poisson family are given as the log odds ratio, where their estimated differences, standard error (*SE*), Z-value and adjusted *p*-value are shown in the table.
The sign after the sample ID "+" or "−" indicates the metal treatment or control, respectively. The genotype ID was considered a random effect in GLMMs. *A. lyrata* samples were excluded from this statistical analysis because they could not survive under the field condition after the metal treatment.
p-values were adjusted by Tukey's honest significance difference.

difference in accumulation level of Zn between *A. halleri* and *A. lyrata* was much larger (more than 100 times) compared to that of Cd (less than five times). In addition, different loci have been isolated for Cd accumulation besides the one locus shared between Cd and Zn accumulation, which suggests that different loci could contribute to divergent accumulations of Zn and Cd (Willems et al., 2010). In *A. kamchatica*, respective loci for Zn and Cd accumulation might have undergone distinct selection after polyploidization. In previous studies, a selective sweep on the key gene for Zn accumulation, *heavy metal ATPase 4* (*HMA4*), was detected in the *halleri* homeolog in both *A. halleri* (Hanikenne et al., 2013) and *A. kamchatica* (Paape et al., 2018), which is consistent with the accumulation levels we obtained here. Nonetheless, a detailed investigation of Cd accumulation is required to genetically explain the lower accumulation level of Cd found in *A. kamchatica*. This may reveal distinct selective pressures on Zn accumulation and Cd accumulation in the tetraploid.

4.2 | Reduced effect of metal accumulation on herbivory in the allotetraploid

A positive effect of metal accumulation on herbivory was detected in the allotetraploid *A. kamchatica* as well as in *A. halleri* in the field experiment. As expected, the effect of metal treatment on herbivory in *A. kamchatica* was lower than in *A. halleri*. This suggests that the elemental defense trait in a hyperaccumulator (*A. halleri*) was attenuated by the allopolyploidization with a non-hyperaccumulator (*A. lyrata*), and this could be attributed to Zn accumulation. Although the effects of metal treatment on herbivory were consistent between the two *A. kamchatica* genotypes in the second week, the outcome of defensive effects became the opposite in the third week between the two genotypes (Table S4). Even when this between-genotype variation was considered a random effect in GLMMs (Bolker et al., 2009), we found significant and positive effects of the metal treatment on herbivory on *A. kamchatica* (Table 3). Previous studies on *A. halleri* suggested that the effectiveness of elemental defense differs between herbivore types (Kazemi-Dinan et al., 2014; Stolpe et al., 2017) and these herbivores differentially respond to other defensive traits, such as glucosinolates (Buckley et al., 2019; Stolpe et al., 2017) and trichomes (Sato & Kudoh, 2015). These defensive traits may exert joint effects on herbivory, because metal accumulation is known to alter glucosinolate concentration in *A. halleri* (Stolpe et al., 2017). Considering the fact that the two *A. kamchatica* genotypes differed in their locality and habitat (Table 1), genetic differentiation in multiple defense traits within the allotetraploid species might be an interesting focal point for future studies.

Besides the elemental defense against herbivory, another plausible positive effect of metal accumulation in *A. kamchatica* was a lower mortality level in the field experiment, especially in kam1 (Figure S4). Metal accumulation is known to cause oxidative damage to cellular molecules, such as membrane lipids and DNA (Schutzendubel & Polle, 2002). Some hyperaccumulators, including *A. halleri*, are known to undergo upregulation in their antioxidation-related genes, most probably to alleviate the metal-induced oxidative damage (Chiang, Lo, & Yeh, 2006; Wójcik, Skórzyńska-Polit, & Tukiendorf, 2006). Thus, the metal treatment might have worked as an acclimation step for mitigating oxidative stress, making the plants more tolerant to later oxidative stress induced by strong sunlight and higher temperatures in the field setting. Although we could not separate the effects of elemental defense and oxidative stresses upon plant viability, our field experiment did show an adverse fitness impact of the metal treatment on the allopolyploid.

Overall, we observed the largest elemental defense effect in *A. halleri*, and the effect on *A. kamchatica* was lower than that on *A. halleri*. This supports the idea that metal accumulation in *A. kamchatica* could occur in a range sufficient to confer elemental defense. As thresholds for the definition of hyperaccumulation, amounts of >3,000 μg g^{-1} of Zn and >100 μg g^{-1} Cd in leaves are often used (Krämer, 2010). Consistent with a herbivory experiment in which >1,000 μg g^{-1} of Zn was shown to function effectively as an elemental defense in *A. halleri* (Kazemi-Dinan et al., 2014), here we found that 1,000 μg g^{-1} of Zn in treated *A. kamchatica* can reduce the level of leaf herbivory, although it is lower than the previously mentioned threshold of hyperaccumulation.

4.3 | Evolutionary and ecological significance of metal accumulation in *A. kamchatica*

Similar to *A. halleri*, the metal contamination levels in natural habitats of *A. kamchatica* are expected to be highly variable given the wide geographic distribution of this species (Paape et al., 2020). Furthermore, genetic variation in ability to accumulate metal among populations should exist in *A. kamchatica*, as already found in *A. halleri* (Babst-Kostecka et al., 2018; Macnair, 2002; Stein et al., 2017). Actually, large variation in Zn accumulation among several natural populations of *A. kamchatica* has been reported from Japan, which included the locality of our kam2 population's origin (Kosugi, Nishizawa, Kawabe, &

Harada, 2016). Our study using two *A. kamchatica* genotypes also detected a variation in accumulation level. The next questions to ask and address would be how the elemental defense is utilized by *A. kamchatica* in its native habitats, and how evolutionary history has shaped the potential variation among populations.

Finally, the variation in other anti-herbivore traits (e.g., glucosinolate biosynthesis, trichome or leaf structure) should affect herbivory, and thus would also be an interesting focal point in further study of the evolutionary history and habitat adaptation of this allopolyploid species. The *A. halleri* and *A. lyrata* species are both well known for having wide natural variation among their respective populations in terms of glucosinolate levels (Buckley et al., 2019; Clauss, Dietel, Schubert, & Mitchell-Olds, 2006; Heidel, Clauss, Kroymann, Savolainen, & Mitchell-Olds,- 2006; Windsor et al., 2005) and other crucial traits against herbivory (Kubota et al., 2015; Stein et al., 2017). Similar genetic variation is also expected among populations of *A. kamchatica*, as a consequence of both selection processes and pre-existent genetic diversity inherited from progenitors through multiple polyploidization origins (Paape et al., 2018; Shimizu-Inatsugi et al., 2009).

ACKNOWLEDGEMENTS

We thank to Ms Aki Morishima for plant preparation and other experimental support. We thank to Ms Midori Takemura, Ms Taeko Tawara and Ms Miwa Yoshikawa for their support with the metal measurements. We appreciate the support from the Zurich Biology Undergraduate Summer School (BUSS) administrator, Ms Audrey Peter, and BUSS's support for S.M.'s stay in Zurich. This study was supported by the University Research Priority Programs (URPP) "Evolution in Action" and "Global Change and Biodiversity" at the University of Zurich, the Japan Science and Technology Agency (JST) (PRESTO, JPMJPR15Q7 and JPMJPR17Q4, to K.T. and Y.S., respectively; and CREST, JPMJCR16O3, to K.K.S.), Swiss National Science Foundation (31003A_182318 to K.K.S), and the Japan Society for the Promotion of Science (MEXT KAKENHI, 16H06469 and 18H04785, to K.K.S.).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

R.S.I. and Y.S. designed the study. R.S.I., K.K.S., and Y.S. wrote the manuscript. G.S.S. and K.T. refined the manuscript. S.M. collected and analyzed herbivory data under supervision by R.S.I. and Y.S. Y.S. performed the statistical analyses. G.S.S. provided plant material. K.T. conducted the metal concentration analysis.

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How to cite this article: Shimizu-Inatsugi R, Milosavljevic S, Shimizu KK, Schaepman-Strub G, Tanoi K, Sato Y. Metal accumulation and its effect on leaf herbivory in an allopolyploid species *Arabidopsis kamchatica* inherited from a diploid hyperaccumulator *A. halleri*. *Plant Species Biol*. 2021;1–10. https://doi.org/10.1111/1442-1984.12304